Short Communication

Supplementation of extracellular adenosine 5'-triphosphate improves sperm post-thawed quality in Thai native chickens

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Abstract

Thai native chicken meat is a high-quality protein source of unique flavor. Genetic preservation and breeding programs are beneficial to maintain purebred populations. Aiming at improving post-thawed sperm quality***, this study is the first to apply*** extracellular adenosine 5'-triphosphate ***(ATPe)*** in freezing extender to preserve Thai native chicken sperm. Eight semen samples from eight Thai native chickens (n = 8) were included. Initial semen evaluation and sperm size measurement were performed. Beltsville poultry semen extender supplemented with 0 and 30 mM ATPe was served as a control and treatment group, respectively. After thawing, motility and motion characteristics were evaluated by computer-assisted sperm analysis system (CASA). Viability and mitochondrial membrane potential were also evaluated. The results demonstrated that the sperm motility (14.35 ± 12.67 vs. 20.41 ± 14.22%, P = 0.038), average path velocity (VAP) (3.58±2.77 µm/sec vs. 5.38±3.75 µm/sec, P = 0.015), curvilinear velocity (VCL) (9.49±6.54 µm/sec vs. 13.27±6.67 µm/sec, P = 0.007) and viability (29.28 ± 6.33 vs. 36.31 ± 9.91%, P = 0.031) were higher in the samples containing 30 mM ATPe compared to the controls. However, the mitochondrial activity was not different. In conclusion, supplementation with 30 mM ATPe clearly improved post-thawed Thai chicken semen quality. Using Thai native chickens as a model, this study will help to improve semen cryopreservation in other endangered avian species.

Keywords: Adenosine 5'-triphosphate, avian, cryopreservation, sperm motion

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Introduction

Thai native chickens (*Gallus domesticus*) are important for meat consumption and cockfighting sport in Thailand (Akaboot *et al*., 2012). They are popular among Thai consumers because the meat quality is distinctly tastier than broilers with high nutrient components (Lengkidworraphiphat *et al*., 2020).Thai native chickens are the descendants of Thai red jungle fowl (*Gallus gallus murghi*) which have become an endangered species and can be found only in national parks and forested mountains (Mekchay *et al*., 2014). Because freezing of bird oocytes and embryos is not possible (Woelders, 2021), sperm cryopreservation is considered to be an alternative assisted reproductive technology for preserving the genetic diversity of these endangered species (Rakha *et al*., 2016). Moreover, sperm cryopreservation and artificial insemination (AI) are worth performing in breeding industries to maintain breeding lines (Thelie *et al*., 2018). AI is reported to be successful in Thai native chickens (Thananurak *et al*., 2020). Although several studies have attempted to find optimal semen cryopreservation protocols for the Thai native chicken (Thananurak *et al*., 2017; Thananurak *et al*., 2019; Thananurak *et al*., 2020; Khunkaew *et al*., 2021), none of them has used extracellular adenosine 5'-triphosphate (ATPe) in the semen extender.

Since avian spermatozoa are measured longer length than other species (Santiago-Moreno *et al*., 2016; de Sousa Barbosa *et al*., 2019), their sperm have been susceptible to morphological disruption during the freezing and thawing. The damaged sperm mitochondria and swollen midpiece (Miranda *et al*., 2018) have contributed to an inability to maintain ATP content after cryopreservation (Long, 2006). A 40-folds decrease in sperm ATP content has been reported in the turkey, contributing to a decline in sperm motility after frozen-thawed (Slowinska *et al*., 2018). Although the ATP production pathway in the avian is poorly understood, it is believed that mitochondrial ATP synthesis plays an important role in maintenance of sperm motility rather than glycolysis (Asano and Tajima, 2017). Also, ATP is the principal energy support of sperm function, i.e. sperm transport in the female reproductive tract, sperm hyperactivation and oocyte penetration (Thuwanut *et al*., 2015). ATP is produced by mitochondrial oxidation of fatty acids and pyruvate, playing an essential role in mammalian oocyte and embryo development (Bradley and Swann, 2019). Increases in sperm viability and motility have been observed in semen extender supplemented with 30 mM ATPe in the cranes and turkeys, respectively (Blanco *et al*., 2011). Moreover, ATPe supplementation has shown benefits on post-thawed epididymal sperm quality of the mouse (Rodríguez-Miranda *et al*., 2008) and rat (Yamashiro *et al*., 2010); and ejaculated sperm of the cat (Thuwanut *et al*., 2015) and the Indochinese leopard (Thuwanut *et al*., 2017).

Since sperm lengths in avian species are varied (Santiago-Moreno *et al*., 2016), morphometry of sperm cell dimension, particularly the sperm head, will allow accurate setting of CASA to differentiate sperm and debris in the samples (de Sousa Barbosa *et al*., 2019). Sperm morphometry using CASA has been performed in many domestic species, such as dogs (Núñez-Martinez *et al*., 2005), mice (Tablado *et al*., 1998), boars (García-Herreros *et al*., 2006), bulls (Vicente-Fiel *et al*., 2013) and cats (de Sousa Barbosa *et al*., 2019). The objectives of this study were to evaluate the impact of ATPe supplementation in sperm freezing extender and to perform morphometric measurement of Thai native chicken spermatozoa.

Materials and Methods

Chemical substance: The chemical substances were purchased from Sigma Chemical Co., St. Louis, MO, USA, unless otherwise stated. Beltsville poultry semen extender II (Continental plastic Corp., USA) was used according to the manufacturer's protocol. Ethylene glycol (8%, v/v) was prepared as a permeating cryoprotectant according to the previous study (Miranda *et al*., 2018). Extracellular adenosine 5'-triphosphate (ATPe) was prepared by adding distilled water to reach a concentration of 30 mM.

***Animals*:** The project protocol was approved by Chulalongkorn University Animal Care and Use Committee (no. 2031086). Eight Thai native chickens aged between 1 to 3 years were included. They were housed individually and fed with a commercial grain diet (CP fighting cock, Charoen Pokphand Foods, Samutprakarn, Thailand). Water was provided ad libitum.

***Semen collection*:** Eight ejaculates (n = 8) of a proven sire were collected from eight Thai native chickens by performing dorsal-abdominal massage (Burrows and Quinn, 1937).The cloaca area of each rooster was cleaned by normal saline solution to avoid contamination of feces. An Eppendorf (1.5 ml) was used as an ejaculate container. Immediately after ejaculation, fresh semen was macroscopically evaluated and mixed with Beltsville poultry semen extender II in 1:1 volume ratio, then transported to the laboratory under 4 °C within 2 hours.

***Semen evaluation*:** Immediately after samples arrived in the laboratory, sperm motility was subjectively evaluated on a warmed glass slide under 400X phase-contrast microscope. Sperm concentration was assessed using a hemocytometer. Samples containing sperm motility more than 70% and concentration more than 90 million sperm cells per ml were included. Sperm viability and morphology were evaluated using eosin-nigrosin staining. Two hundred sperm were classified into two categories: live sperm were unstained whereas dead sperm were stained red. Mitochondrial membrane potential was evaluated by fluorochrome 5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazolyl -carbocyanine iodide (JC-1; Molecular Probe Inc.) staining, then assessed under fluorescent microscope (Olympus corporation, Tokyo, Japan). Two hundred spermatozoa were evaluated for mitochondrial membrane integrity and classified into two categories: high (stained with bright orange through sperm midpiece) and low mitochondrial membrane potential (stained with patchy pale orange

or all green through sperm midpiece) Percentages of live sperm and sperm with high mitochondrial potential were calculated. Sperm morphology: head length and width, and tail length were measured by CellSens Standard software (Olympus corporation, Tokyo, Japan) under 1000X magnification. Ten randomized morphologically normal spermatozoa from each individual rooster were measured for head and tail length. Head length was measured from the tip of the sperm head to the junction next to the midpiece. Head width was measured from one side of the head parallel to another side of the head at the widest region. Tail length was measured from the end of the midpiece to the end of the tail (Fig. 1). The head length and width and tail length were recorded.

Freezing and thawing procedure: The procedure was modified from the previous study (Miranda *et al*., 2018). Briefly, the semen was diluted with Beltsville poultry semen extender II to the final concentration of 60 x 10^6 sperm per ml and cooled at 5 °C for 30 mins. Thereafter, 8% ethylene glycol was added as a permeating cryoprotectant. ATPe (Sigma-Aldrich, St.Louis, MO, USA) at 0 and 30 mM concentration was prepared as a control and treatment, respectively. After equilibration at 5 °C for 45 mins, samples were loaded into 0.25-ml plastic straws. The straws were frozen by being placed horizontally at 5 cm above liquid nitrogen vapor for 15 mins in a Styrofoam box and plunged into liquid nitrogen. After the samples were stored for up to 1 week, thawing was performed according to the previous study (Miranda *et al*., 2018). The straws were immersed in a water bath at 5 °C for 2 mins prior to emptying them into 1.5-ml Eppendorfs.

***Sperm motion analysis*:** Sperm motion characteristics were evaluated after thawing using Sperm Class Analyzer CASA system (Microptic S.L, Barcelona, Spain). Sperm motility, progressive motility (PM) and motion characteristics including average path velocity (VAP), straight line velocity (VSL) and curve linear velocity (VCL) were recorded.

***Statistical analysis*:** All data was analyzed using IBM SPSS Statistics for Windows (Version 22.0. Armonk, NY: IBM Corp.). Percentages of motility, PM, VAP, VSL, VCL, viability and mitochondrial membrane potential were compared between treatment and control group using paired t-test. The level of significance was set at $P < 0.05$.

Results and Discussion

All of the ejaculates had a milky macroscopic appearance. The volume of the ejaculates was between 300 to 1500 µl. The percentages of motility, viability and mitochondrial activity of fresh sperm were 76.86 ± 9.4, 82.78 ± 8.54 and 72.31 ± 22.9% respectively. The average dimension of the Thai native chicken spermatozoa (n = 80) was revealed (Table 1, Figure 1). The sperm dimension was in the range according to the previous report (*Gallus domesticus*) (Santiago-Moreno *et al*., 2016). The post-thawed sperm quality of the control and treatment group was demonstrated (Table 2). After post-thawing, the sperm motility, VAP, VCL, and viability were higher in the samples containing 30 mM ATPe than the controls ($P < 0.05$). This result is in accordance with the research in turkeys, cranes (Blanco *et al*., 2011) and the other mammals (Yamashiro *et al*., 2010; Thuwanut *et al*., 2015; Thuwanut *et al*., 2017). ATPe supplementation to the semen extender significantly provided higher motility than those without. Interestingly, the mitochondrial membrane potential did not differ between groups which was contrary to the cats (Thuwanut *et al*., 2015) and Indochinese leopards (Thuwanut *et al*., 2017).

Table 1 Dimension of Thai native chicken sperm measured by CellSens Standard software (mean ± SD, n = 80 spermatozoa)

Parameter	Average size (µm)
Head length	14.45 ± 1.64
Head width	1.11 ± 0.14
Tail length	80.80 ± 6.63

Table 2 Sperm parameters of Thai native chicken spermatozoa treated with 0 (control) or 30 mM extracellular adenosine 5'-triphosphate (ATPe) (mean ± SD) (n = 8 chicken)

Parameter	Control	ATPe	P -value
Motility (%)	14.35 ± 12.67	20.41 ± 14.22	0.038*
Progressive motility (%)	1.48 ± 1.88	2.61 ± 4.15	0.222
VAP (µm/s)	3.58 ± 2.77	5.38 ± 3.75	0.015*
VSL (µm/s)	1.76 ± 1.63	2.83 ± 2.84	0.078
VCL (µm/s)	9.49 ± 6.54	13.27 ± 6.67	0.007*
Viability (%)	29.28 ± 6.33	36.31 ± 9.91	0.031*
Mitochondrial integrity (%)	11.31 ± 9.05	16.22 ± 11.23	0.114

*Value with superscript indicates significant difference (P<0.05)

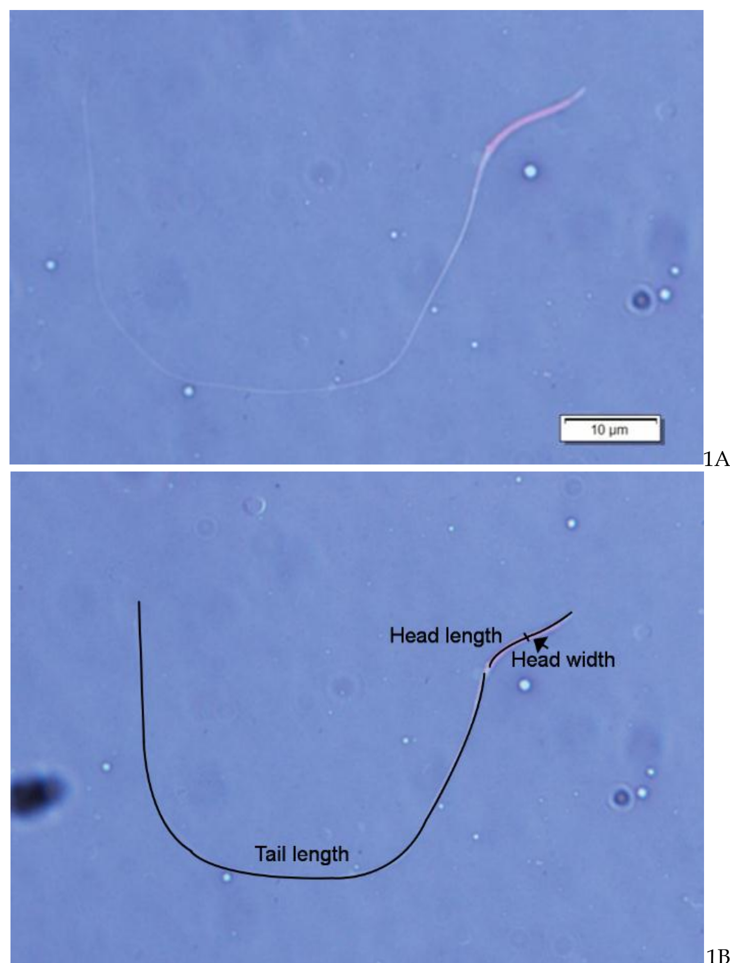

Figure 1 Measurement of head and tail length and head width of chicken sperm (1A, photo and 1B, diagram illustration)

ATPe was reported to stimulate P2 purinoceptor with a high affinity and induce calcium uptake by the sperm in a dose-dependent manner (Luria *et al*., 2002). Thus, it facilitated hyperactive mice sperm movement resulting in significant increases of VCL, VSL, and VAP (Rodríguez-Miranda *et al*., 2008). Accordingly, this study demonstrated ATP induced increases of VCL and VAP in the chicken sperm. In addition, ATPe stimulated non-membrane soluble adenylyl cyclase (SACY) leading to an increase of intracellular cAMP and the calcium level through the pyruvate dehydrogenase activation in the Krebs cycle. This mechanism directly provided ATP for sperm flagella movement (Thuwanut *et al*., 2015). As the sperm ATP concentration was reported as being strongly correlated with fertility (Wishart and Palmer, 1986), adding of ATPe to the semen freezing extender likely improves the potential of assisted reproductive technology in Thai native chickens as well as other endangered avian species.

In conclusion, this is the first report of sperm dimension in the Thai native chicken. In addition, supplementation of Beltsville poultry semen extender with 30 mM ATP obviously improves post-thawed sperm motility, VAP, VCL, and viability. This study will help to improve the sperm freezing protocol for Thai native chickens and other endangered avian.

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